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Award Number: DAMD17-97-1-7287

TITLE: Methyl-Deficient Diets and Risks of Breast Cancer Among
African-American Women: A Case-Control Study by
Methylation Status of the ER Gene

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REPORT DATE: January 2001

TYPE OF REPORT: Final

PREPARED FOR: U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

DISTRIBUTION STATEMENT: Approved for Public Release;
Distribution Unlimited

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20011005 320

REPORT DOCUMENTATION PAGE

Form Approved
OMB No. 074-0188

Public reporting burden for this collection of information is estimated to average 1 hour per response, including the time for reviewing instructions, searching existing data sources, gathering and maintaining the data needed, and completing and reviewing this collection of information. Send comments regarding this burden estimate or any other aspect of this collection of information, including suggestions for reducing this burden to Washington Headquarters Services, Directorate for Information Operations and Reports, 1215 Jefferson Davis Highway, Suite 1204, Arlington, VA 22202-4302, and to the Office of Management and Budget, Paperwork Reduction Project (0704-0188), Washington, DC 20503

1. AGENCY USE ONLY (Leave blank)		2. REPORT DATE January 2001	3. REPORT TYPE AND DATES COVERED Final (25 Sep 97 - 24 Dec 00)	
4. TITLE AND SUBTITLE Methyl-Deficient Diets and Risks of Breast Cancer Among African-American Women: A Case-Control Study by Methylation Status of the ER Gene			5. FUNDING NUMBERS DAMD17-97-1-7287	
6. AUTHOR(S) Kangmin Zhu, M.D., Ph.D. Nasar U. Ahmed, Ph.D.				
7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES) Meharry Medical College Nashville, Tennessee 37208-3599 E-MAIL: zhukak75@yahoo.com			8. PERFORMING ORGANIZATION REPORT NUMBER	
9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES) U.S. Army Medical Research and Materiel Command Fort Detrick, Maryland 21702-5012			10. SPONSORING / MONITORING AGENCY REPORT NUMBER	
11. SUPPLEMENTARY NOTES				
12a. DISTRIBUTION / AVAILABILITY STATEMENT Approved for public release; distribution unlimited				12b. DISTRIBUTION CODE
13. ABSTRACT (Maximum 200 Words) This is the final report of our case-control study testing the hypothesis that methyl-deficient diets are more likely to be related to breast cancer with methylated CpG islands of the estrogen-receptor (ER) gene. Cases were 304 African-American patients pathologically diagnosed with breast cancer during 1995-1998 and who lived in three counties, Tennessee. Controls were 305 African-American women without breast cancer, who were selected through random-digit dialing and frequency matched to cases by 5-year age range and county. Information on dietary intake and other risk factors were collected through telephone interviews. Dietary methyl-components were defined based on dietary folate and methionine intake and alcohol consumption. Tumor tissue samples were collected for the measurement of methylation status of the ER genes. Our preliminary results showed that the odds ratio (OR) estimates for lower folate intake were 1.53 (95% confidence interval (CI), 0.60-4.04) for cases with methylated genes, 0.46 (95%CI, 0.18-1.16) for cases with un-methylated genes, and 1.40 (95%CI 0.61-3.20) for cases with unknown methylation status (presumably including cases with both methylated and un-methylated genes). No consistent results were obtained for methionine intake and alcohol use. Although somewhat suggestive, these preliminary results did not show a coherent pattern coinciding with the study hypothesis.				
14. SUBJECT TERMS Breast Cancer, Case-Control Study, ER Gene, Methyl-Deficient Diets, Alcohol, Methylation				15. NUMBER OF PAGES 44
				16. PRICE CODE
17. SECURITY CLASSIFICATION OF REPORT Unclassified	18. SECURITY CLASSIFICATION OF THIS PAGE Unclassified	19. SECURITY CLASSIFICATION OF ABSTRACT Unclassified	20. LIMITATION OF ABSTRACT Unlimited	

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INTRODUCTION

This is the final report of our case-control study entitled "Methyl-Deficient Diets and Risk of Breast Cancer among African-American Women: A Case-Control Study by Methylation Status of the ER Genes". This study was aimed to examine whether the relationship between methyl-deficient diets and breast cancer differs depending upon the methylation status of the estrogen receptor (ER) genes in African-American women, based on a study hypothesis we developed. To reach our study goal, we conducted a case-control study, in which we needed to enroll breast cancer cases and controls, interview with study subjects for information about dietary intake and other factors, collect tumor tissue specimens of the cases, measure methylation status of the ER genes, analyze data from epidemiological investigation and laboratory measurement, and write a scientific report.

BODY

RESEARCH ACCOMPLISHMENT

With the collective effort of the research team, the support of the Tennessee Cancer Reporting System (TCRS), the collaboration of doctors and pathology departments of the hospitals in the study area, and the help from Dr. Nancy Davidson at Johns Hopkins

University, we accomplished the tasks outlined in the Statement of Work. These tasks primarily included: identifying cases and their doctors and seeking consent from them; recruiting controls through random digit telephone dialing; interviewing study subjects; collecting tumor tissue specimens; isolating DNA from tumor samples; measuring methylation status of the ER genes and ER status; obtaining information about disease diagnosis from the TCRS; entering and cleaning data; conducting nutrient analysis and other data analyses; and writing the report. Recruitment of and interview with part of the study subjects were supported by another study.

The tasks were accomplished under the condition of three major obstacles that we confronted during research: more obstacles than expected in getting consent from doctors; difficulty in recruiting African Americans into the study; and difficulty in the laboratory measurement of methylation status. With the same funding, we put extra effort into recruiting study subjects, such as more tasks pertaining to obtaining doctors' consent and home visits of eligible African-American women who did not respond to our mails. Home visits substantially increased the participation of eligible women. It is not easy to measure methylation status of DNA samples from paraffin-embedded tissue specimens. When we failed to get satisfactory results after working on it for a long period, Dr. Nancy Davidson from Johns Hopkins University, an expert in this

field, kindly helped perform the laboratory measurement in her laboratory for the project. Her help was invaluable for the completion of the project.

Because the proportions of patients without a doctor identified and doctors who did not respond or did not give consent were higher and the participation rate of eligible women was lower than expected, however, the number of cases enrolled was 77 fewer than that in the proposal. Particularly, a few doctors who had a relatively large number of African-American patients refused to participate in the study. As a result, the pool of patients we could contact was reduced. The other problem for the study was that satisfactory results on methylation status were not achieved for some cases. These may have led to a lower study power. Nevertheless, we still utilized cases with unknown methylation status. The use of these cases as a separate group in data analyses would provide additional information about whether methyl-deficient diets are more likely to be related to breast cancer with methylated ER genes (see below).

The following parts of this report body describe the study and report the preliminary results in a format for a scientific publication.

INTRODUCTION

Risk factor profile of breast cancer may be different according to estrogen receptor (ER) status of tumor. However, previous epidemiological studies have obtained inconsistent results [1-5]. Part of the reason for the inconsistency may be the lack of sharpened hypothesis due to little knowledge on the underlying cause of ER level variation. Recent studies have shown that ER-negative status results from the methylation of the ER gene CpG islands, cytosine- and guanine-rich areas located in the promoter regions of genes [6-7]. Because low dietary methyl-components can cause abnormal DNA methylation including methylation of the CpG islands that are usually unmethylated [8-9], it is reasonable to hypothesize that methyl-deficient diets are more likely to be related to the carcinogenesis of breast cancer with the methylated ER gene CpG islands.

This hypothesis is based on the three theoretical bases. First, CpG island methylation is an early event in carcinogenesis. Second, CpG island methylation of the ER gene may cause low ER expression in breast cancer. Third, methyl-deficient diets (diets deficient in methyl-groups such as methionine and folate) can cause abnormal methylation of genes and therefore may relate to

carcinogenesis.

CpG island methylation as an early event in carcinogenesis has been shown in many studies. In normal adult tissues, CpG islands are unmethylated and are transcriptionally active [10-11]. When CpG islands are methylated, chromatin structure can change, causing genes in these chromosome regions to become inactive in transcription and causing unstable DNA that lead to tumorigenesis. Studies have demonstrated that (1) the methylation of CpG islands increases in tumor tissues and occurs prior to allelic losses of genes [12], (2) the methylation increases with age in normal tissues [13], and (3) the occurrence of the methylation precedes oncogene-induced transformation [11]. These findings suggest that CpG island methylation appears early in carcinogenesis.

Lack of ER expression has been well linked to the methylation status of CpG islands in the ER genes located on chromosome 6q24-q27 [14,15]. An earlier study showed that the lack of ER gene expression is related to methylation of the 5' region of the ER gene [16], as shown that the region were hypermethylated in 4 out of 5 ER-negative carcinomas and hypomethylated in 13 of 15 ER-positive carcinomas [17]. Recent studies demonstrated that the methylation of CpG island in the 5' region and first exon of the gene is responsible for lack of expression of ER gene in ER-negative breast tumors [6]. The

methylation of CpG island in ER-negative tumors was confirmed by the reactivation of ER gene expression by demethylation of the ER gene [7]. The results based on breast cancer specimens also showed that ER-negative tumors have higher mean scores of ER CpG island methylation than ER-positive tumors [18].

Studies have found that diets deficient in methionine and folate or high in methyl group antagonists (such as alcohol) can cause abnormal methylation of genes. Studies in animals showed that the CpG sites were methylated during the transition to tumor after a relatively long-term methyl-deficient diet [8]. The methylation of usually unmethylated CpG sites may result from increased DNA methyltransferase (DNA-MTase) activity induced by methyl-deficient diets [19]. The hypermethylation of the CpG islands silences tumor suppressor genes [20] such as the ER gene [21] and therefore is related to the occurrence of cancer. Methyl-deficient diets may also simultaneously cause global genomic hypomethylation by decreasing cellular levels of the methyl donor S-adenosylmethionine [22-26]. The global genomic hypomethylation can activate some oncogenes [27]. Decreased S-adenosylmethionine can also facilitate the activity of DNA-MTase as a mutator enzyme, leading to CpG mutagenesis [24]. Probably as a result of these DNA changes, diets reducing methyl-group availability may increase the risk of cancer, as observed in animal [25,28] and human studies [19,29].

Integrating these theoretical associations, we postulate that methyl-deficient diets may be more likely to be related to breast-cancer where the ER CpG islands are methylated. Because the methylation of usually unmethylated CpG sites may be associated with low dietary methyl-components, tumors with the methylated ER gene may be especially susceptible to the effects of methyl-deficient diets and methyl-antagonists. On the other hand, these dietary factors may be less influential and other risk factors may be important for tumors without the methylation.

Previous epidemiological studies suggest the importance of testing this hypothesis. Without information on methylation status of the ER genes, studies on fruit/vegetables (rich in folate) and poultry/fish/dairy products (rich in methionine) have found either an association between lack of these dietary factors and increased risk of breast cancer [30-34], or no association [35,36]. Studies on alcohol consumption (a methyl group antagonist) also have showed a null or weak positive association with breast cancer [37-39]. These results suggest that there may be an association, but the association might be diluted due to the lumping of tumors with different epigenetic characteristics. Therefore, studies distinguishing patients with different methylation status and measuring methyl components instead of food items are needed to

clarify the relationship. In the present investigation, we examined the relationship between methyl-deficient diets and breast cancer according to the methylation status of the ER gene CpG islands among African-American women aged 20-64.

MATERIALS AND METHODS

Study Subjects

We conducted a case-control study to test our hypothesis. Cases were 304 African-American female patients pathologically diagnosed with breast cancer during 1995-98 and who lived in Davidson, Shelby and Hamilton counties, Tennessee. Controls were 305 African-American women without a history of breast cancer, who were selected through random-digit telephone dialing and frequency matched to cases by 5-year age range and county.

Cases were selected through the Tennessee Cancer Reporting System (TCRS). TCRS periodically provided us with a list of eligible patients, their mailing addresses, their doctors and their doctors' addresses and telephone numbers. We sent a letter and a consent form to the doctors for their consent to contact their patients. The letter we sent described the study and asked if we could contact their patients. A second mail was mailed to

physicians who did not respond to the first one. If a physician did not return the consent form after two mails, one of our staff members called the physician's office to determine the status of the letter and faxed or mailed another copy of the letter and consent form when needed.

Patients with a doctor's consent were sent a cover letter and a consent form for their participation in the study. The letter introduced the study procedures and a woman's right as a study participant and asked if they would participate in the study. The second packet was mailed to those who did not respond to the first one. A reminder call (where a telephone was available) was made to women who did not reply to both mailings. For women who did not respond and did not have a telephone listed, we sent a nurse, a breast cancer survivor, a social worker or a research assistant with African-American ethnicity to their homes to seek their consent. Only patients who completed a consent form were recruited as cases for the study.

We selected controls using random digit dialing techniques [40]. We first grouped cases diagnosed in the same calendar year whose telephone area codes serve the same county, and then formed the sampling frame by age distribution of the cases in the area, using an eligibility table. By randomly selecting one of the

telephone prefixes of the cases and adding the last four random-selected digits, a call was made to find an eligible woman according to ethnic background and age range.

For each telephone number called, interviewers determined (1) whether it was a residential or nonresidential (business line, cellular network, fax machine, disconnected, or changed to another number,) number; (2) whether there were any eligible women for a residential number; and (3) how many eligible women there were (randomly select one, if more than one eligible women). Up to 9 calls over a two-week period, including 3 day-time, 3 evening, and 3 weekend calls, were made for a telephone number that was not answered. If an eligible woman was identified, we described the study purposes and procedures, and asked whether she would accept a telephone interview. For a woman who agreed to participate, a telephone interview was conducted.

To achieve a high response rate, we used a monetary incentive for both cases and controls (\$25 for a completed interview and a drawing for an award of \$200). We also provided cases with \$10 for their agreement to release their tumor tissue specimens.

TCRS provided us with 645 eligible breast cancer patients with a doctor identified. A doctor's consent for us to contact was obtained

for 480 patients (76%). Out of the 480 patients, 18 deceased and 50 were unable to locate. Three hundred and four cases (63% of those with a doctor's consent) agreed to participate in the study and were subsequently interviewed.

We identified 385 women eligible as frequency-matched controls. Out of the women, 305 (79.2%) participated in the study.

Collection of Epidemiological Data

Telephone interview technique was used to collect information on dietary intake and other breast cancer risk factors. Telephone interviewers were trained on conversation skills on the telephone, how to conduct an interview based on the questionnaire, and the avoidance of inappropriate questioning and inferring. They were asked to examine completed questionnaires immediately after an interview for any errors, inconsistencies, unusual answers and missing values, and to make corrections or compensations where possible.

Information collected included dietary intake, demographic variables, reproductive and menstrual history, medical history, family history of cancer, personal habits (smoking, alcohol consumption and exercise), and anthropometric variables (weight and

height).

Methyl-deficient diets were the exposure of interest. We estimated dietary intake using the Block-NCI Health Habits and History Questionnaire [41]. The questionnaire includes foods representing at least 90 percent of the total U.S. consumption of each of 18 major nutrients. It asks the detailed information about the frequency of use and commonly serving size for each item of fruits and juices, breakfast foods, vegetables, animal meats, breads, snacks, spreads, dairy products, sweets and beverages. It also collects information about the use of supplemental vitamins. Nutrient estimates for the dietary assessment are computed based on the NHANES II nutrient content database. This database is based on U.S. Department of Agriculture food composition data tapes, as well as industry and other sources. Dietary data were analyzed to calculate nutrient estimates, using the Dietary Analysis Personal Computer System (DIETSYS). Dietary methyl-content was defined by methionine and folate intakes.

Other information we collected included (1) reproductive factors and menstrual status (age at menarche, number of parity, number of full-term pregnancies, age at first and last full-term pregnancies, spontaneous and induced abortion, total months of breast feeding, menopausal status, and age at menopausal); (2)

medical history (benign breast diseases, sexually-transmitted diseases, and cancers in other sites); (3) family history of breast cancer (breast cancer in mother, sisters, daughters and aunts, and their ages at diagnosis); (4) personal habits and medication use (alcohol consumption, cigarette smoking, use of electric bedding devices, oral contraceptive and other exogenous estrogen use); (5) anthropometric measures (height and weight (1-2 years before reference date and at age 18); (6) detection mode of breast cancer (for cases only) (how the disease was detected); and (7) demographic variables (date of birth, age at diagnosis of breast cancer (for cases), marital status, usual occupation, education level, religion preference, annual family income and the number of family members).

Tumor tissue collection and laboratory measurement

Paraffin-embedded tumor tissue samples were collected from hospitals where cases were pathologically diagnosed. Tissue slides were made. A pathologist reviewed slides for each sample. Tissue samples without tumor identified were excluded. DNA from the tissue samples was extracted using a method described originally by Sukpanichnant et al. [43] with some modifications. We first cut four 10 micron thick sections and collected them into 1.5 ml microcentrifuge tube. Tissue sections were deparaffinized in xylene * 2 changes for 30 minutes. The cells were released from the paraffin at this stage. Then we washed them in absolute ethanol for

2 changes, 10 minutes each, digested them in solution of 50 mM Tris PH 8.5, 1mM EDTA, 0.5% Tween 20 and proteinase K (200ug/ml) for 3 hours or overnight at 50°C. In the following morning, the samples were boiled in digest solution for 10 minutes, quickly spun to remove the supenatant and then transferred to a fresh sterile tube and stored at -20°C.

Using extracted DNA samples, PCR method [18,44] was chosen to assess methylation patterns in the 5' CpG island of the ER gene. The PCR method is based on the principle that unmethylated cytosine residues in DNA are converted to uracil when treated with sodium bisulfite, while methylated residues are not converted [45]. Therefore, the sequence of the treated DNA differs depending on if the DNA is originally methylated or unmethylated. Using this concept, primers were designed to detect methylation patterns in the 5' region of the ER gene. Primer pairs for PCR amplification were purchased from BRL Life Technologies (Gaithersburg, MD) [18]. For this study we used primer 5 for detection of unmethylated and methylated CpG islands. The primer pairs were

```
ER5 u          5'-GGTGTATTTGGATAGTAGTAAGTTTGT-3'
                5'-CCATAAAAAAACCAATCTAACCA-3'
ER5 m          5'-GTGTATTTGGATAGTAGTAAGTTCGTC-3'
                5'-CGTAAAAAAAACCGATCTAACCG-3'
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We used CpGenome DNA modification kit from Oncor company to

perform the chemical modification of DNA samples. Isolated and treated DNA were then subjected to PCR amplification using the buffer prepared according to Lapidus et al's article [18]. Reactions were done for 37 cycles in a Perkin-Elmer 9600 thermocycler. As a control untreated DNA were also amplified from the same subjects. PCR amplification products were separated and visualized on 3.0 % agarose gels stained with EtBr.

CpG methylation status was determined according to whether there was a PCR product produced in the methylated or unmethylated reaction and visible by ethidium bromide staining. Reactions were considered to be negative when no positive bands are shown and positive when positive bands exist. Positive bands were scored from one (weak) to three (strong). Methylated CpG islands were defined when a positive methylated band was found. Unmethylated CpG islands were determined when no methylated reactions were identified and a positive unmethylated band was found. Methylation status was unable determined for some samples because no PCR bands were shown due to insufficient quantity or unsatisfactory quality of DNA samples.

Data Analysis

In data analysis, we compared cases with methylated ER genes, cases with unmethylated ER genes with controls, respectively. Except these 3 comparison groups, an additional group of cases, for whom

methylation status was unable to be measured, was also employed. With unknown methylation status, this group included cases both with and without methylated ER genes. Therefore, we assumed that the association strength for this group should have lied between that for methylated cases and that for unmethylated cases if methyl-deficient diets or methyl-antagonists are more likely to be related to breast cancer with methylated ER genes.

Nutrient assessment was an important part of the analysis. DIETSYS generates estimates for different nutrients. Folate intake could be calculated automatically by DIETSYS. Methionine intake was estimated by using information on its content from each food item [42], which was entered into the database. Information on the amount of alcohol consumption was also from the Block-NCI questionnaire. The frequency and serving size of beer, wine and liquor consumption were asked. We assigned 12.8 g of alcohol for each 12 oz can of beer, 13.8 g of alcohol for each medium glass of wine, and 14.0g of alcohol for each shot of liquor. A weight of 0.5 was given to the serving size smaller and 1.5 was given to the serving size larger than those specified above. The frequency was recoded according to the DIETSYS manual. We estimated consumption for each type of alcohol (beer, wine and liquor) by multiplying the number of grams with recoded frequency. Average daily alcohol consumption was computed by summing consumption from all three types and was divided by 14 to get the average number of drinks per day.

Before conducting any analyses, we excluded women with

improperly completed forms. These included 17 cases and 15 controls who reported unusually low or high dietary kilocalories (<500/day or >4000 per day) or who reported a relatively large number of foods with missing data or eaten per day (>30 foods).

As the first step of data analysis, we described the distribution of demographic characteristics for four comparison groups. The descriptive analyses of other factors in terms of the disease status were also conducted.

Logistic regression was used to assess the relationship between methyl-deficient diets and breast cancer [46]. Three case groups were compared with the control group. The exposure variables compared were methyl-deficient diets (dietary folate and methionine) or methyl antagonist (alcohol). We first used dichotomized exposure variables determined based on the 50 percentile of the distribution. We also used quartile variables to assess if there was a dose-response relation. To control for potential confounders, we always put in the models demographic variables such as age, marital status, educational level, and annual family income. Total energy intake and vitamin intake were also kept in the model because of possible correlation between these factors and methyl-deficient diets and because of the potential effects of these factors on breast cancer. Other factors were selected into the model based on whether they were related to breast cancer in the descriptive analysis and whether they could theoretically confound the relationship between methyl-deficient diets or alcohol and breast cancer. The odd ratio (OR) for

a risk factor and its 95% confidence interval (CI) were estimated in the analyses.

RESULTS

Table 1 shows demographic characteristics of study subjects. Cases with methylated ER genes tended to be older, compared with other case groups and controls. They were more likely to have had some college education compared with controls, while cases with unmethylated ER genes or with unknown methylation status were more likely to be college or professional-school graduates. All case subgroups tended to have higher household income than controls.

These demographic variables and other potential confounders were adjusted when assessing the relationship between methyl-deficient diets or alcohol consumption and breast cancer. Table 2 presents the odds ratio estimates of dietary intakes of folate and methionine and alcohol consumption for breast cancer. For folate, the OR estimates for having lower intake ($\leq 443.9\mu\text{g/day}$) were 1.53 (95%CI, 0.60-4.04) and 0.46 (95%CI, 0.18-1.16) for methylated cases and un-methylated cases, respectively, while the corresponding estimate lay between them for cases with unknown methylation status. For methionine, the ORs for lower intake ($\leq 0.78\text{g/day}$) were 1.05 (95%CI, 0.45-2.49) and 0.61 (95%CI, 0.25-1.51) for methylated and un-methylated cases, and the estimate was higher for cases with

unknown methylation status (OR=1.60, 95%CI, 0.72-3.53). The OR estimates for alcohol consumption were between 0.6 and 0.8 for all three case groups.

Table 3 shows the results when data were analyzed according to the quartiles of folate and methionine intakes, the number of years of alcohol consumption, and the amount of alcohol consumed. The risk of breast cancer with methylated genes tended to increase with decreasing folate intake although the trend was not significant (p for trend >0.05). A similar, but less obvious trend was seen for tumors with unknown methylation status. However, the risk seemed to be lower as folate intake decreased for cases with un-methylated genes. For methionine, although the risk of breast cancer tended to be higher for lower methionine intake compared with the highest intake level for cases both with methylated genes and with unknown methylation status, an increasing risk with decreasing intake was shown only for the latter. The risk tended to be lower with decreasing methionine intake for cancer with un-methylated genes. Compared with women who did not drink or drank for less than 10.5 years (25th percentile), women who drank for longer than 26 years tended to have a higher risk of breast cancer for the methylated group. A higher amount of alcohol consumption (>0.5 drinks/day) tended to increase the risk of all types of breast cancer. The increase appeared more obvious for cases with methylated ER genes.

However, again, the confidence intervals of the ORs contained one.

DISCUSSION

We reported the results based on our preliminary data analyses. More analyses will be conducted. The preliminary results on folate showed that the OR estimates seemed greater than one for cases with methylated genes, smaller than one for cases with un-methylated genes, and between the two values for cases with unknown methylation status. The dose-relation (increased risk with decreasing folate intake) seemed more obvious for cases with methylated genes. Although this pattern appeared to be consistent with our study hypothesis, the confidence intervals of the OR estimates contained the unity and the p-values for trends were not statistically significant. The results on the amount of alcohol consumption and methionine showed tendencies that a larger amount of alcohol intake and a long period of drinking may be more likely to be associated with breast cancer with methylated ER genes and the OR estimates for methionine intake appeared higher for cases with methylated genes and with unknown methylation status when comparing the highest level of intake. However, the comparison of three case groups in the OR estimates did not show a consistent pattern coinciding with the study hypothesis. There may be several study limitations that may partly account for the inconsistency of the results.

Insufficient study power could be an explanation of our results. Because about forty percent of cases did not have information available on methylation status of their cancer, it substantially reduced the number of cases with and without methylated ER genes. The reduced number of study subjects may have caused decreased study power. Dietary factors generally do not have a strong association with cancer. For example, the deficiency of methyl-components in human diets is unlikely to be as severe as that in animal studies [25] and DNA methylation may only lead to increased susceptibility to cancer [47]. Therefore, the association of methyl-deficient diets with breast cancer may be weak or moderate. A relatively small number of study subjects usually brings about a wide confidence interval of an OR estimate and is unable to reveal such a weak or moderate association. Therefore, despite the interesting pattern on folate intake, we are unable to ascertain an association supporting our study hypothesis.

Dietary assessment was based on the intake in the year prior to the reference date. However, the effect of nutrient on carcinogenesis usually occurs many years before diagnosis of cancer. Therefore, it is remote diet that is of interest for etiologic studies of cancer. Although commonly used, diet in the year prior to the reference date may not be a good surrogate of remote diet. The measurement errors may have had impact on our results. The

other potential problem in dietary assessment is that information from cases may have been affected by the change in diets due to the diagnosis of cancer [48]. While epidemiological studies on dietary factors in relation to cancer are frequently influenced by these innate drawbacks, our study might not be an exception. However, the influences, if any, might not be differential between the three case groups. Therefore, the relative differences between the case groups might not be substantially biased.

Except for the commonality in their impact on methylation of genes, folate, methionine and alcohol may be involved in other different biological mechanisms and may interact differently with other factors. For example, folate is also essential for the synthesis of purines and the pyrimidine nucleoside thymidine and folic acid deficiency may destabilize the DNA molecule, causing malignant transformation [49]. Alcohol may also contain a small amount of carcinogens and its metabolites may influence the metabolism and DNA damaging effect of xenobiotics and endogenous compounds, relating to carcinogenesis [50]. Due to the effects of these mechanisms, the associations of folate, methionine and alcohol with breast cancer may appear with variation among them.

We used PCR primer 5 to define the methylation status in this study. There are other primers available for the measurement, such

as primers 1, 3 and 4 [18]. Although primer 5 is a good choice, results based on only one primer might not be completely correlated with the true methylation status. If misclassification on methylation status existed (it is unlikely to be differential by dietary intake status), the differences between cancers with and without methylated ER genes would have been diluted. We do not exclude this possibility.

A substantial proportion of eligible women did not participate in the study because of no doctors identified, no doctor's consent available, or no consent obtained from the women. If the non-participating patients differed systematically from the participating cases in the intake of folate, methionine or alcohol and such differences were differential in terms of methylation status of the ER genes, the results might be biased. Although this differentiation in differences is unlikely, we cannot exclude such a possibility.

This is the first study that examines risk factors according to the epigenetic characteristics of breast cancer. Despite the limitations of the study and despite the fact that, based on our preliminary data analyses, we could not draw a conclusion on whether methyl-deficient diets are more likely to be associated with breast cancer with methylated CpG islands of the ER genes, some of the

results may suggest the necessity of further research. More analyses of our data will be conducted soon.

KEY RESEARCH ACCOMPLISHMENTS

- Obtained consents from doctors and patients through mailing, calling and home visiting, and interviewed cases;
- Randomly called more than 15,000 telephone numbers to identify controls and interviewed eligible controls;
- Collected tumor tissue specimens from hospitals in the three study counties;
- Isolated DNA from tissue samples and measured methylation status of the ER gene of specimens;
- Entered and cleaned data and did preliminary analysis; and
- Published and submitted two manuscripts.

REPORTABLE OUTCOMES

1. Submitted manuscript entitled "African-American ethnicity in epidemiological studies of calcium antagonists in relation to cancer"
2. Published article, "Methyl-deficient diets, methylated ER genes and breast cancer: an hypothesized association"
3. A proposed project based on the data from this study, "African-

American Women with the CYP1A1 MspI and African-American-Specific Polymorphisms May Have Different Risk Factor Profile of Breast Cancer", has been recommended for the Concept Award by the Department of Defense.

CONCLUSIONS

Few population-based epidemiological studies on breast cancer have been conducted in Tennessee and no population-based case-control studies have been done by Meharry Medical College, a minority institution. We had to make great effort to establish and maintain a system for such a population-based study. We had to cope with barriers to obtaining doctors' consent, barriers to recruiting African Americans into the study, and difficulties in the laboratory measurement of methylation status. Considering these obstacles, our research team has been successful in reaching the goal indicated in the proposal. Because no studies on the topic have been done, results from our study have provided initial data on whether methyl-deficient diets are more likely to be related to breast cancer with methylated ER genes. The data we obtained have laid a solid fundamental for the development of more research projects. We deeply appreciate the Department of Defense Breast Cancer Research Program for the support of this study and appreciate the technical reviewers of the proposal and annual reports for their comments.

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APPENDICES

1. Table 1. Demographic characteristics of study subjects
2. Table 2. Odds ratio estimates of dietary folate intake, dietary methionine intake and alcohol intake in relation to breast cancer
3. Table 3. Dose-relation of dietary folate intake, dietary methionine intake and alcohol intake to the risk of breast cancer
4. Published article

Table 1. Demographic characteristics of study subjects, Davidson, Hamilton and Shelby counties, Tennessee, 1995-1998

Variable	Controls	ME cases	UM cases	UK cases
<i>Age at interview</i>				
20-39	31(%10.7)	12(%14.0)	11(%12.8)	9(%7.8)
40-49	99(%34.0)	35(%40.7)	24(%27.9)	39(%33.6)
50-59	107(%36.8)	27(%31.4)	34(%39.5)	40(%34.5)
>=60	54(%18.6)	12(%14.0)	17(%19.8)	28(%24.1)
<i>Marital status at reference date</i>				
Married	126(%43.6)	38(%44.2)	41(%47.7)	47(%40.5)
Separated	32(%11.1)	10(%11.6)	10(%11.6)	10(%8.6)
Divorced	60(%20.8)	20(%23.3)	19(%22.1)	32(%27.6)
Widowed	33(%11.4)	5(%5.8)	6(%7.0)	11(%9.5)
Never-married	38(%13.2)	13(%15.1)	10(%11.6)	16(%13.8)
<i>Employment at reference date</i>				
No	91(%31.5)	25(%29.1)	28(%32.6)	35(%30.2)
Yes	198(%68.5)	61(%70.9)	58(%67.4)	81(%69.8)
<i>Education level</i>				
<=High school	130(%44.8)	31(%36.1)	33(%38.4)	47(%40.5)
Vocational school	28(%9.7)	11(%12.8)	6(%7.0)	15(%12.9)
Some college	75(%25.9)	30(%34.9)	21(%24.4)	22(%19.0)
College Graduate or professional school	54(%18.6)	13(%15.1)	26(%30.2)	32(%27.6)
Other	3(%1.0)	1(%1.2)	0(%0.0)	0(%0.0)
<i>Religion</i>				
None	11(%3.8)	4(%4.7)	4(%4.7)	6(%5.2)
Protestant	261(%90.0)	77(%90.6)	74(%86.1)	98(%84.5)
Catholic	7(%2.4)	2(%2.4)	1(%1.2)	5(%4.3)
Other	11(%3.8)	2(%2.4)	7(%8.1)	7(%6.0)
<i>Household Income (dollars)</i>				
<15,000	102(%36.6)	25(%30.1)	18(%22.2)	38(%33.3)
15,000-29,999	82(%29.4)	27(%32.5)	16(%19.8)	18(%15.6)
30,000-44,999	54(%19.4)	12(%14.5)	18(%22.2)	32(%28.1)
>=45,000	41(%14.7)	19(%22.9)	29(%35.8)	26(%22.8)

Table 2. Odds ratio estimates of dietary folate intake, dietary methionine intake and alcohol intake in relation to breast cancer by methylation status of the ER genes, Davidson, Hamilton and Shelby counties, Tennessee, 1995-1998

Factor	ME cases (n=79)		UM cases (n=81)		UK cases (n=109)	
	OR*	95%CI**	OR	95%CI	OR	95%CI
<i>Dietary folate intake</i>						
>443.9µg/day	Reference		Reference		Reference	
≤443.9µg/day	1.53	0.60-4.04	0.46	0.18-1.16	1.40	0.61-3.20
<i>Dietary methionine intake</i>						
>0.78g/day	Reference		Reference		Reference	
≤78g/day	1.05	0.45-2.49	0.61	0.25-1.51	1.60	0.72-3.53
<i>Alcohol consumption</i>						
No	Reference		Reference		Reference	
Yes	0.74	0.38-1.44	0.63	0.34-1.18	0.78	0.45-1.36

Abbreviation: ME, methylated; UM, un-methylated; UK, unknown methylation status.

* Adjusted for age, employment status, marital status, educational level, income, number of people household, religion, smoking, use of electric blanket/mattress pad, menopausal status, use of estrogen, use of progesterone, history of benign breast disease, family history of breast cancer, weight, height, physical activity, number of pregnancies, number of miscarriages, on a diet to lose weight, having an infertility test, dietary intake of vitamin B2, B6 and C, supplemental folate intake, and values of total energy intake per day; ** 95% confidence interval.

Table 3. Dose-relation of dietary folate intake, dietary methionine intake and alcohol intake to the risk of breast cancer by methylation status of the ER genes, Davidson, Hamilton and Shelby counties, Tennessee, 1995-1998

Factor	ME cases (n=79)		UM cases (n=81)		UK cases (n=109)	
	OR*	95%CI**	OR	95%CI	OR	95%CI
<i>Dietary folate intake</i>						
>612.8µg/day	Reference		Reference		Reference	
443.9-612.8µg/day	0.68	0.24-1.89	1.54	0.54-4.40	0.74	0.30-1.81
325.7-443.8µg/day	1.08	0.29-4.05	0.76	0.19-3.01	1.04	0.33-3.30
<=325.6µg/day	1.40	0.24-7.77	0.52	0.09-3.06	1.15	0.26-5.04
		p ^s >0.05		p>0.05		p>0.05
<i>Dietary methionine intake</i>						
>1.06g/day	Reference		Reference		Reference	
0.79-1.06g/day	1.75	0.69-4.43	1.23	0.47-3.26	1.21	0.54-2.70
0.55-0.78g/day	1.75	0.56-5.52	0.72	0.22-2.34	1.66	0.62-4.43
<=0.54g/day	1.41	0.34-5.76	0.74	0.17-3.16	2.85	0.84-9.71
		p>0.05		p>0.05		p>0.05
<i>Number of years alcohol-drunk</i>						
<=10.5 years	Reference		Reference		Reference	
10.6-20 years	1.74	0.71-4.28	0.48	0.17-1.38	0.96	0.41-2.26
20.1-26 years	0.27	0.05-1.46	0.80	0.24-2.67	1.17	0.40-3.37
>26 years	2.17	0.65-7.29	1.34	0.49-3.65	1.19	0.44-3.24
		p>0.05		p>0.05		p<0.05
<i>Amount of alcohol consumed***</i>						
None	Reference		Reference		Reference	
<=0.5 drinks/day	0.92	0.46-1.84	1.16	0.59-2.27	1.48	0.84-2.63
>0.5 drinks/day	3.63	0.98-13.42	2.80	0.88-8.93	2.11	0.68-6.52
		p>0.05		p>0.05		p>0.05

Abbreviation: ME, methylated; UM, un-methylated; UK, unknown methylation status.

* Adjusted for age, employment status, marital status, educational level, income, number of people household, religion, smoking, use of electric blanket/mattress pad, menopausal status, use of estrogen, use of progesterone, history of benign breast disease, family history of breast cancer, weight, height, physical activity, number of pregnancies, number of miscarriages, on a diet to lose weight, having an infertility test, dietary intake of vitamin B2, B6 and C, supplemental folate intake, and values of total energy intake per day; ** 95% confidence interval; *** Based on the consumption in the year preceding the reference date; ^s, p for trend.

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Methyl-deficient diets, methylated ER genes and breast cancer: An hypothesized association

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(Received 22 May 1998; accepted in revised form 13 August 1998)

Recent molecular studies show that ER-negative breast cancer results from the lack of ER gene transcription due to the methylation of the CpG island 5' to the gene. Because CpG island methylation is an early event in carcinogenesis and because methyl-deficient diets could result in CpG island methylation, it is relevant to postulate that methyl-deficient diets may be a risk factor for breast cancer with methylated ER genes (as opposed to the disease with unmethylated ER genes). This molecular-based etiologic hypothesis may facilitate epidemiological research on the relationship between breast cancer and diet that has been unclear until now. *Cancer Causes and Control*, 1998, 9, 615-620

Key words: Breast cancer, estrogen receptors, gene, methyl-deficient diets, methylation.

Introduction

Breast cancer can be divided into two types according to the tumor estrogen receptor (ER) level: ER-positive or ER-negative. Because the presence or absence of ERs in breast cells may differentially affect the role some risk factors, such as estrogens, play on the etiology of the disease, it is reasonable to hypothesize that risk factor profiles of breast cancer vary by ER status of the disease. However, previous epidemiological studies on risk factors by ER status have obtained inconsistent results.¹⁻⁸ Recent molecular studies show that ER-negative breast cancer results from the lack of ER gene transcription due to the methylation of the CpG island 5' to the gene.^{9,10} We suggest that this observation may be critical in assessing breast cancer risk factors according to the ER status of the tumor.

The inconsistency in previous epidemiological studies by ER status may be related to problems in using total

ER levels as an indicator of ER status without fully understanding the basis of ER level variation. Moreover, it is possible to misclassify an individual's ER status by just measuring total ER levels. For example, a tumor with a sparse distribution of ER-positive cells may be falsely considered ER-negative and a tumor with a dense distribution of ER-negative cells may be falsely considered ER-positive. This patchiness or variegation and failure to understand the underlying cause of ER level variation may have affected study results and conclusions.

Using the methylation status of the ER genes is less likely to be prone to the same effects of cellularity in defining ER status, and may help define a molecular-based etiologic hypothesis of breast cancer. Because CpG island methylation is an early event in carcinogenesis and may relate to breast cancer's lack of ER

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expression and because diets deficient in methyl-groups (such as methionine, choline, and folate) can result in abnormal DNA methylation/carcinogenesis, it is reasonable to postulate that methyl-deficient diets may be a risk factor for breast cancer with methylated ER genes, but not for the disease with unmethylated ER genes.

CpG island methylation is an early event in carcinogenesis

CpG islands are located in the promoter regions of genes and their methylation status is important in gene transcription.¹¹⁻¹³ Active transcription requires an unmethylated state of 5' sites that exist in normal adult tissues.^{12,14,15} When CpG islands are methylated, chromatin structure can change, causing genes in these chromosome regions to become transcriptionally inactive.¹⁶ These chromosome alterations may also result in DNA instability leading to tumorigenesis. In a study on colon cancer, Makos *et al.*¹⁶ found that there is abnormal methylation of the CpG island areas on 17p in colon adenomas and the abnormality increases in colon cancers. Because allelic losses of chromosome 17p are characteristics of colon carcinomas, the results suggest that methylation precedes these allelic losses. Another study of colorectal tumors¹⁴ showed that CpG island methylation of the ER gene increases with age in human colonic mucosa from normal individuals and can be found in all colorectal tumors. Vertino *et al.*¹⁵ examined whether the aberrant methylation of CpG islands evolves as a function of immortalization and oncogene-induced neoplastic transformation of bronchial epithelial cells. They found that the methylation of CpG islands at 17p13 occurred during the immortalization of normal human bronchial cells and preceded oncogene-induced transformation. Because chromosome 17p13 deletions occur in lung tumorigenesis,¹⁷ the results suggest that the methylation appears early in bronchial epithelial cell carcinogenesis that is related to immortalization.¹⁵ In addition, Vertino *et al.*¹⁵ found that aberrant CpG island methylation appeared during normal aging of fibroblasts and may predispose some cells to transform into cancer. Combined, these studies imply that CpG island methylation is an early event of carcinogenesis.

CpG island methylation of the ER gene may cause low ER expression in breast cancer

The human ER gene is located on chromosome 6q24-q27.^{18,19} Recent studies have shown that ER-negative breast cancer is caused by a lack of ER gene transcription.^{18,20} The lack of ER gene expression is related to methylation of the 5' region of the gene:²¹ 4 out of 5

samples were hypermethylated in ER-negative carcinomas and 13 of 15 were hypomethylated in ER-positive carcinomas.²² Using human breast cancer cell lines, it was subsequently demonstrated that methylation of the CpG island in the 5' region and first exon of the gene is responsible for lack of expression of ER gene in ER-negative breast tumors.⁹ This was confirmed by reactivating the ER gene using inhibitors of DNA methylation, which demethylate the ER CpG island.¹⁰ Although the results based on breast cancer specimens are more complex due to the heterogeneity of cell populations within a tumor, it was found recently that ER-negative tumors have higher mean scores of ER CpG island methylation than ER-positive tumors.²² By analogy to the colorectal cancer story given above, ER gene methylation may be an early event in some breast cancer (*i.e.* ER negative), if breast cancer shares similar molecular mechanisms to other tumors.

Risk factors may differ depending upon the methylation status of the ER genes

Certain risk factors may be important for tumors with methylated genes and other factors may be significant for other tumors. For example, in a recent study,²³ it was found that lung cancers from smokers and from animals exposed to tobacco-specific carcinogens had a low incidence of CpG island methylation of the ER genes, while lung cancers of non-smokers and spontaneous tumors in animals had a high incidence of methylation. For breast cancer, it can be postulated that factors that can cause or facilitate CpG island methylation of the ER gene may only increase the risk of breast cancer with ER gene methylation. Due to a lack of receptors resulting from the methylation, breast cells with methylated CpG islands may not be affected by subsequent exposures to estrogens during their transformation into cancer cells. On the contrary, tumors with unmethylated ER genes, and therefore with receptors, may be more susceptible to factors that can interact with ERs. If these differences exist, breast cancers with and without ER gene methylation will have distinct risk factor profiles.

Methyl-deficient diets could result in abnormal DNA methylation and therefore are more likely to be related to breast cancer where the ER gene CpG islands are methylated

No studies have been conducted on breast cancer risk factors according to the methylation status of the ER gene. However, the possibilities discussed above imply an association of methyl-deficient diets with breast cancers where the ER gene is methylated. Such an

association, if it exists, may be based on the following hypothesized mechanisms. It is suggested that diets deficient in methyl-groups (such as methionine and folate) or high in methyl group antagonists (such as alcohol) cause increased DNA methyltransferase (DNA-MTase) activity.²⁴ There may be two types of DNA-MTase activities: *de novo* methyltransferase activity and maintenance methyltransferase activity.²⁵ Elevated *de novo* DNA-MTase activity may initiate^{25,26} and elevated maintenance DNA-MTase activity may subsequently spread and maintain²⁶ methylation of the usually unmethylated CpG sites, possibly through the disruption of the boundaries that normally protect CpG islands from methylation.²⁶ Methylation of the CpG sites after a relatively long-term methyl-deficient diet has been directly demonstrated during the transition to tumor in animals,²⁷ although it was not suggested in a study in humans,²⁸ in which short-term dietary methyl group restriction was used and methylated urine metabolites rather than methylation of the CpG sites was measured. The hypermethylation of the CpG islands silences tumor suppressor genes²⁹ such as the ER gene³⁰ and therefore is related to the occurrence of cancer. Methyl-deficient diets can also lower cellular levels of the methyl donor S-adenosylmethionine.³¹⁻³³ Reduced S-adenosylmethionine can cause global genomic hypomethylation^{25,32,34,35} and therefore the activation of some oncogenes.²⁶ Decreased S-adenosylmethionine can also facilitate the activity of DNA-MTase as a mutator enzyme, leading to CpG mutagenesis.²⁵ Probably as a result of these DNA changes, diets reducing methyl-group availability may increase the risk of cancer. Observations in animal models³⁶ and humans^{24,37,38} support this. In Giovannucci *et al.*'s study,²⁴ a combination of high alcohol and low methionine and folate intake conferred a relative risk of 7.4 for distal colon cancer. Because low dietary methyl-components may cause (1) the methylation of ER gene CpG islands that reduces tumor suppressing activities of the ER genes, (2) global genomic hypomethylation that may activate some oncogenes and (3) CpG mutagenesis, it is reasonable to hypothesize that methyl-deficient diets and those high in methyl-antagonists are likely to be related to breast cancer primarily with methylated ER genes. Figure 1 depicts the hypothesized association.

Our hypothesis of the association between methyl-deficient diets and breast cancer with ER gene methylation suggests the need to study breast cancer risk factors with respect to specific molecular characteristics. Tumors with and without a specific molecular characteristic may have different causal pathways and therefore have different risk-factor profiles. The differences may originate from two things. First, the change in methylation pattern is probably not inherited. Rather, it

may result from a number of environmental or somatic factors that do not co-occur in cancers without this molecular change. Second, even though methylation changes exist (due to either environmental exposures or somatic factors), they may not cause cancer alone. It is likely that methylation imparts susceptibility to cells and causes cancer in the presence of other genetic or environmental factor(s). Because these other factors have their effects in conjunction with this susceptibility, their association with cancer would be different, depending upon whether a tumor has such susceptibility.

Several issues should be considered in the exploration of the relationship between methyl-deficient diets, breast cancer and methylation status of the ER gene. First, methyl-deficient diets are also associated with global genomic hypomethylation related to the occurrence of cancer. If the hypomethylation could occur without methylation of CpG islands, breast cancer without methylated ER genes may also be susceptible to the effects of methyl-deficient diets. While we do not exclude this possibility, it is unlikely because widespread genomic hypomethylation and methylation of CpG islands usually exist simultaneously in tumor cells.²⁶ Second, the metabolism of methyl groups is influenced by methylenetetrahydrofolate reductase (MTHFR).^{39,40} A mutation in the MTHFR gene, which is common in many populations,⁴¹ can reduce specific MTHFR activity, leading to decreased methionine and S-adenosylmethionine levels.⁴² Decreased S-adenosylmethionine in individuals with the MTHFR mutation appears only in the presence of low folate status.⁴³ Therefore, the association between methyl-deficient diets or methyl-antagonists and cancer might be stronger among people with mutated MTHFR genes, as suggested by recent studies.^{44,45} The effect of methyl-deficient diets on breast cancer with methylated ER genes, if any, may be modified by the MTHFR genotype, which should be considered in studies on the hypothesized association. Finally, the hypothesized association between methyl-deficient diets and the risk of breast cancer with methylated ER gene is based on the hypothesis that breast cancers with and without methylated ER genes are two different entities that may have different etiologic pathways. This hypothesis is tenable because the methylation status of CpG islands has been suggested as an early event in the development of cancer. However, if methylated CpG islands also occur as a function of tumor progression after a tumor develops, they may appear in some late-stage breast cancers that were unmethylated at their early stage, leading to the misclassification of real methylation status. Early-stage tumors should be used if this is true.

Many epidemiologic studies of cancer risk factors have not distinguished tumors by genetic or epigenetic

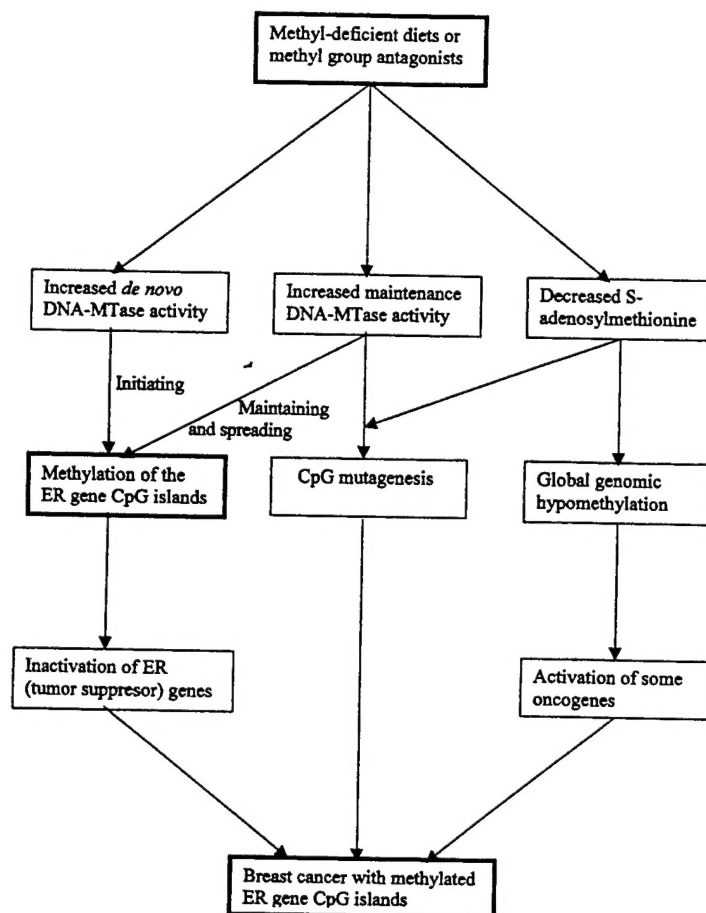


Figure 1. Hypothesized association between methyl deficient diets and breast cancer with methylated ER gene CpG islands.

characteristics.⁴⁶ The pooling of similar cancers having different causal pathways would dilute the ability to detect risk factors for each pathway. Without information on the methylation status of ER gene CpG islands, previous epidemiological studies on fruits/vegetables (rich in folate^{24,37}) and poultry/fish/dairy products (rich in methionine²⁴) have found either an association between the lack of these dietary factors and increased risk of breast cancer,⁴⁷⁻⁵¹ or no association.^{52,53} Studies on alcohol consumption (a methyl group antagonist) also have showed a null or weak positive association with breast cancer.⁵⁴⁻⁵⁶ Because methyl-deficient diets and methyl group antagonists are related to abnormal DNA methylation, they may be a risk factor for tumors with methylated ER genes, but not for those without. The lumping of tumors with different ER gene methylation statuses may have led to an estimate of a diluted association. Case-control studies on methyl-deficient diets, in which breast cancers are distinguished by the methylation status of the ER genes, can be used to explore such a possibility. Cohort studies are also

feasible by examining the ER methylation status of tumors among women with and without methyl-deficient diets. Studies that distinguish different genetic or epigenetic status of tumors would improve research on the relationship between risk factors and the disease,^{57,58} increasing our ability to comprehend diet-breast cancer relationships that have not been clear to this point.

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